BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

As rescanning documents *will not* correct images, please do not report the images to the Image Problem Mailbox.

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
B01L 3/00

A1

(11) International Publication Number: WO 97/36681

(43) International Publication Date: 9 October 1997 (09.10.97)

(21) International Application Number: PCT/US97/05458

(22) International Filing Date: 2 April 1997 (02.04.97)

(30) Priority Data:
60/014,712 3 April 1996 (03.04.96) US

(71) Applicant: THE PERKIN-ELMER CORPORATION [US/US]; 850 Lincoln Centre Drive, Foster City, CA 94404 (US).

(72) Inventors: WOUDENBERG, Timothy, W.; 120 Arbor Lane, Moss Beach, CA 94038 (US). ALBIN, Michael; 5127 Longdale Court, Antioch, CA 94505 (US). KOWALLIS, Reid, B.; 2875 Canyon Road, Burlingame, CA 94010 (US). RAYSBERG, Yefim; 1157 Vuelta Olivos, Fremont, CA 94539 (US). RAGUSA, Robert, P.; 14404 DeBell Road, Los Altos, CA 94022 (US). WINN-DEEN, Emily, S.; 239 Stilt Court, Foster City, CA 94404 (US).

(74) Agents: POWERS, Vincent, M. et al.; Dehlinger & Associates, P.O. Box 60850, Palo Alto, CA 94306-0850 (US).

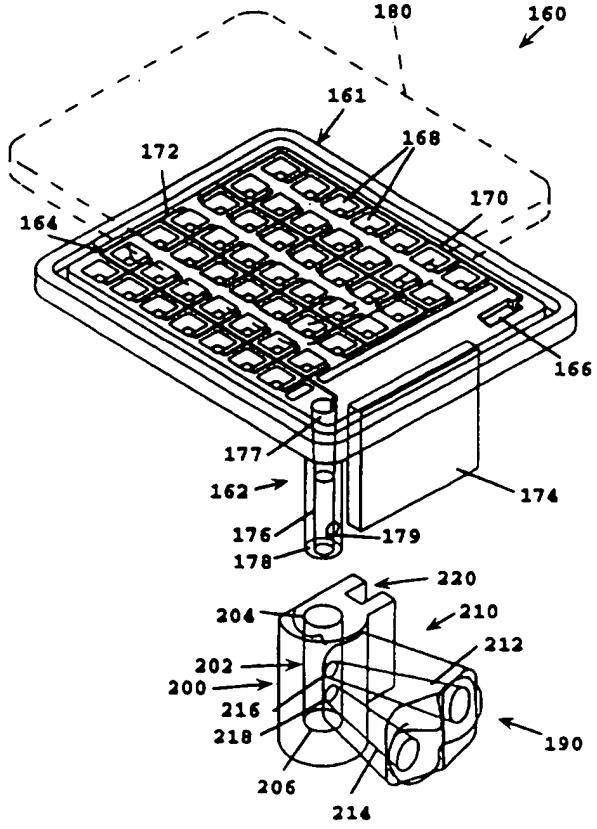
(81) Designated States: AU, CA, JP, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: DEVICE AND METHOD FOR MULTIPLE ANALYTE DETECTION

(57) Abstract

The invention is directed to a method and device for simultaneously testing a sample for the presence, absence, and/or amounts of one or more a plurality of selected analytes. The invention includes, in one aspect, a device for detecting or quantitating a plurality of different analytes in a liquid sample. The device includes a substrate which defines a sample-distribution network having (i) a sample inlet, (ii) one or more detection chambers, and (iii) channel means providing a dead-end fluid connection between each of the chambers and the inlet. Each chamber may include an analyte-specific reagent effective to react with a selected analyte that may be present in the sample, and detection means for detecting the signal. Also disclosed are methods utilizing the device.



DEVICE AND METHOD FOR MULTIPLE ANALYTE DETECTION

Field of the Invention

The present invention relates to devices and methods for detecting or quantifying one or more selected analytes in a sample.

References

Ausubel, F. M., et al., Current Protocols in Molecular Biology, John Wiley & Sons, Inc., Media, PA.

Bergot, J.B., et al., PCT Pub. No. WO 91/05060 (1991).

Blake, et al., Biochemistry 24:6132 (1985a).

Blake, et al., Biochemistry 24:6139 (1985b).

Buchardt, O., et al., PCT Pub. No. WO 92/20703 (1992).

Froehler, et al., Nucl. Acids Res. 16:4831 (1988).

Fung, S., et al., EPO Pub. No. 233,053 A2 (1987).

Higuchi, R., et al., Bio/Technology 10:413 (1992).

Higuchi, R., et al., Bio/Technology 11:1026 (1993).

Ishiguro, T., et al., Anal. Biochem. 229:207 (1995).

Kornberg, A., et al., DNA Replication, pp 46-47, W.H. Freeman and Co., New York (1992).

Landegren, U., et al., Science 241:1077 (1988).

Lee, L.G., et al. Nucl. Acids Res. 21:3761 (1993).

Livak, K.J.., et al. PCR Methods and Applications 4:357 (1995).

Menchen, S.M., et al., U.S. Pat. No. 5,188,934 (1993).

Menchen, S.M., et al., PCT Pub. No. WO 94/05688 (1994).

Miller, P.S., et al, Biochemistry 18:5134 (1979).

Miller, P.S., et al., J. Biol. Chem. 255:6959 (1980).

Miller, P.S., et al., Bioconj. Chem. 1:187 (1990).

Mullis, K., U.S. Patent #4,683,202 (1987).

Murakami, et al., Biochemistry 24:4041 (1985).

Saiki, R.K., et al., Science 230:1350 (1985).

Sambrook, J., et al., Molecular Cloning, 2nd Ed., Cold Spring Harbor Laboratory Press, NY (1989).

Segev, D., PCT Pub. No. WO 90/01069 (1990).

Segev, D., "Amplification of Nucleic Acid Sequences by the Repair Chain Reaction" in Nonradioactive Labeling and detection of Biomolecules, C. Kessler (Ed.), Springer Laboratory, Germany (1992).

inlet. Preferably, each chamber includes an analyte-specific reagent effective to react with a selected analyte that may be present in the sample, and detection means for detecting the signal.

In one embodiment, the detection means for each chamber includes an optically transparent window through which the signal can be detected optically. In another embodiment, the detection means includes a non-optical sensor for detecting the signal.

The channel means of the device may be configured in numerous ways. For example, in one embodiment, the channel means includes a single channel to which the detection chambers are connected by dead-end fluid connections. In a second embodiment, the channel means includes at least two different channels, each connected to a different group of detection chambers. In yet another embodiment, the channel means includes an individual channel for each detection chamber.

The device may include a vacuum port for placing the detection chambers under vacuum prior to the addition of sample. In one embodiment, the vacuum port is connected to the channel means at a site between, and in fluid communication with, the sample inlet and the detection chambers. In another embodiment, the vacuum port is connected to the channel means at a site downstream of the detection chambers. In this configuration, the vacuum port is additionally useful for removing liquid from the channel means after the detection chambers have been filled, to help isolate the detection chambers from one another and further reduce cross-contamination.

The vacuum port may be incorporated in a multi-port valve (e.g., a 3-way valve) that permits the network and associated detection chambers to be exposed alternately to a vacuum source, the sample inlet, and a vent or selected gas source.

Alternatively, the device of the invention is prepared and sealed under vacuum when manufactured, so that a vacuum port is unnecessary.

According to an important feature of the invention, the device is capable of maintaining a vacuum within the sample-distribution network (low internal gas pressure, relative to the external, ambient pressure outside the device) for a time sufficient to allow a sample to be drawn into the network and distributed to the detection chambers by vacuum action. For this purpose, the sample-distribution network may include a vacuum reservoir in fluid communication with, and downstream of, the detection chambers, for preventing the build-up of back-pressure in the network while the detection chambers are successively filled.

In one embodiment, the vacuum reservoir includes a non-flowthrough cavity connected downstream of the last-filled detection chamber, for accumulating residual gas displaced from the inlet and channel means. In another embodiment, the reservoir comprises the terminal end of the

In another aspect, the invention includes a method for detecting or quantitating a plurality of analytes in a liquid sample. In the method, there is provided a device of the type described above, wherein the interior of the network is placed under vacuum. A liquid sample is then applied to the inlet, and the sample is allowed to be drawn into the sample-distribution network by vacuum action, delivering sample to the detection chambers. The delivered sample is allowed to react with the analyte-specific reagent in each detection chamber under conditions effective to produce a detectable signal when the selected analyte is present in the sample. The reaction chambers are inspected or analyzed to determine the presence and/or amount of the selected analytes in the sample.

The device of the invention may also be provided as part of a kit which additionally includes selected reagents, sample preparation materials if appropriate, and instructions for using the device.

These and other objects and features of the invention will be more apparent from the following detailed description when read in light with the accompanying drawings.

Brief Description of the Drawings

Figs. 1A and 1B show a plan view (1A) and perspective view (1B) of an exemplary assay device in accordance with the invention;

Figs. 2A-2C illustrate several exemplary sample distribution network configurations in accordance with the invention;

Figs. 3A-3C illustrates a time sequence for the filling of the detection chambers of a sample-distribution network with fluid sample;

Fig. 4 illustrates a sample-distribution network containing three sample delivery channels for delivering sample to three different sets of detection chambers;

Fig. 5 illustrates a sample-distribution network having a separate delivery channel for each detection chamber;

Figs. 6A-6C illustrate selected features of another sample-distribution network in accordance with the invention; the device is shown in plan view (6A), perspective view (6B), with a portion of the sample distribution network of the device shown in Fig. 6C;

Fig. 7 shows an exploded view of a portion of a device in accordance with the invention;

Fig. 8 shows an exploded view of a portion of another device in accordance with the invention; and

Fig. 9 shows a perspective view of another device in accordance with the invention.

Inlet 38 may be adapted to form a vacuum-tight seal with the end of a syringe, for sample loading, or with a multi-port valve to provide fluid communication with the sample and one or more liquid or gaseous fluids. The inlet may further include a septum cap, if desired, for maintaining the network under vacuum and allowing introduction of sample by canula or needle.

Vacuum port 40 may be adapted for connection to a vacuum source, such as a vacuum pump. The vacuum connection may include a valve for closing off the sample-distribution network from the vacuum source, or a multi-port valve for connection to a vacuum source and one or more selected gas supplies.

Substrate 30 further provides indentations or holes 42, which may be arranged asymmetrically as illustrated in Fig. 1A, to engage corresponding pins or protrusions in a device-holder, not shown, to immobilize and orient the device for analysis.

As noted in the Summary of the Invention, the sample-distribution network of the invention may utilize any of a number of different channel configurations, or channel means, for delivering sample to the individual detection chambers. With reference to Fig. 2A, distribution network 34a includes sample inlet 38a, a plurality of detection chambers 44a, and channel means comprising a single channel 46a to which the detection chambers are each connected by dead-end fluid connections 48a. The detection chambers are distributed on either side of channel 44a, with the fluid connections branching off in pairs from opposite sides of the channel. Fig. 2B shows a portion of an alternative network 34b having an inlet 38b and detection chambers 44B, wherein fluid connections 48b branch off from channel 46b in a staggered manner.

The detection chambers in the device of the invention may be arranged to form a repeating 2-dimensional array which facilitates indexing and identification of the various chambers, as well as allowing rapid measurement of an optical signal produced by each chamber upon reaction with the sample, if optical detection is used.

Figs. 2A-2B, for example, show networks in which the detection chambers are arranged in rows and columns along perpendicular axes, allowing the chambers to be identified by X and Y indices if desired. This type of array (a perpendicular array) also facilitates successive interrogations of the chambers in a chamber-by-chamber analysis mode. However, other arrangements may be used, such as a staggered or a close-packed hexagonal array. Fig. 2C, for example, shows part of a network 34c having inlet 38c and an array of staggered detection chambers 44c. The detection chambers are connected to a common delivery channel 46c by fluid connections 48c.

The device may also include identifying symbols adjacent the detection chamber to facilitate identification or confirmation of the analytes being detected.

detection reagents and detected all in the same chamber, without requiring movement of the sample from each chamber to another site. Moreover, since the sample and detection reagents can remain in the chamber for signal detection, the detection reagents need not be immobilized on or adhered to the inner surfaces of the detection chambers.

The components of the sample-distribution network are designed to ensure that an adequate volume of sample will be delivered to the detection chambers to allow accurate analyte detection and/or quantitation. In general, the percent-volume of a detection chamber that must be occupied by the sample will vary according to the requirements of the reagents and the detection system used. Typically, the volume-percent will be greater than 75%, preferably greater than 90%, and more preferably greater than 95%. In assay formats in which the detection chambers are heated, particularly to temperatures of between about 60°C and about 95°C, the volume-percent filling of the chambers is preferably greater than 95%, and more preferably is at least 99%.

The degree to which the detection chambers are filled with sample will generally depend upon (1) the initial ratio of the external (atmospheric) pressure to the initial pressure within the network, (2) the individual and total volumes defined by the detection chambers, (3) the volume defined by the channel means, and (4) the nature of the network downstream of the last detection chamber.

For example, in the case of a detection chamber which is nearest the sample inlet, and which will be filled first, the percentage occupancy (volume-percent) of sample fluid in the chamber after sample loading ($V_{f,s}$) will be related to the external atmospheric pressure (P_{ext}) and the initial internal pressure within the network before sample loading (P_{int}) by the expression:

$$V_{f,s} \approx (P_{ext})/(P_{ext} + P_{int})$$

Thus, if the initial pressure within the network (P_{int}) is 10 mm Hg, and the external pressure (P_{ext}) is 760 mm Hg, about 99% of the first detection chamber will be filled with sample fluid ($V_{f,s} \approx 99\%$), with the remaining volume ($\approx 1.3\%$) being filled by residual gas (e.g., air) displaced by the sample. (This calculation assumes that, by the time the sample reaches the chamber, the internal network pressure has not increased appreciably due to displacement of gas upstream of the chamber.) Similarly, if P_{ext} is 760 mm Hg and P_{int} is only 40 mm Hg, the volume-percent of the chamber that becomes occupied with sample will still be very high (about 95%).

It will be appreciated that as the sample fluid reaches and fills successive detection chambers, the residual gas displaced from the channel means will gradually accumulate in the remaining network volume, so that the internal pressure will gradually increase. The resultant increase in

The sample-distribution network in Fig. 4 further includes separate vacuum reservoirs 70a-70c which are connected to the termini of sample delivery channels 64a-64c, downstream of the detection chambers. The vacuum chambers are dimensioned to help maintain a low internal gas pressure during sample loading.

In another embodiment, the channel means includes an individual channel for each detection chamber, as illustrated in Fig. 5. Network 80 includes an inlet 82, detection chambers 84, and associated with each detection chamber, a dead-end fluid connection 86, which may also be referred to as channel means, for delivering sample to each chamber. Each dead-end fluid connection is dimensioned to define a volume that is substantially less than the volume of the associated detection chamber, to ensure that each detection chamber is sufficiently filled with sample. This embodiment provides rapid filling of the detection chambers with minimal cross-contamination.

The device of the invention may also include a vacuum port communicating with the sample-distribution network, for applying a vacuum to the network before or during sample loading. In one embodiment, the vacuum port is connected to the channel means at a site between, and in fluid communication with, the sample inlet and the detection chambers. An illustration of this can be found in Fig. 9. The vacuum port thus provides a convenient way to reduce the internal pressure within the network to a selected residual pressure prior to sample loading. In particular, when the sample is introduced into the network using a syringe barrel connected to the sample inlet, the vacuum port can be used to remove air from the space between the syringe and the inlet, before the sample is admitted into the network.

In another embodiment, the vacuum port is connected to the channel means at a site downstream of the sample inlet and detection chambers (e.g., Fig. 6A). In this configuration, the vacuum port may additionally be used to remove liquid from the channel means after the detection chambers have been filled, to help isolate the detection chambers from one another and further reduce cross-contamination. In this configuration, the vacuum port constitutes a part of the vacuum reservoir described above, where the reservoir includes a vacuum source linked to the terminal end of a sample delivery channel. The vacuum port may be kept open to the network during sample loading, to continuously remove residual gas from the network until all of the detection chambers have been filled.

The vacuum port may include a multi-port valve (e.g., 3-way valve) that permits the network and associated detection chambers to be exposed alternately to a vacuum source, the sample inlet, and a vent or gas source. Such a valve may be used to alternately expose the network to vacuum and a selected gas source, to replace residual air with the selected gas. Such gas replacement in

that are shallow, deep, square, rectangular, concave, or V-shaped, or any other appropriate configuration.

Typically, the detection chambers will be dimensioned to hold from 0.001 μL to 10 μL of sample per chamber, and, more preferably between 0.01 μL and 2 μL. Conveniently, the volume of each detection chamber is between about 0.1 μL and 1 μL, to allow visual confirmation that the chambers have been filled. For example, a chamber having a volume of 0.2 μL may have dimensions of 1 mm x 1 mm x 0.2 mm, where the last dimension is the chamber's depth.

The sample delivery channels are dimensioned to facilitate rapid delivery of sample to the detection chambers, while occupying as little volume as possible. Typical cross-sectional dimensions for the channels will range from 5 μm to about 250 μm for both the width and depth. Ideally, the path lengths between chambers will be as short as possible to minimize the total channel volume. For this purpose (to minimize volume), the network is preferably substantially planar, i.e., the channel means and detection chambers in the device intersect a common plane.

The substrate that defines the sample-distribution network of the invention may be formed from any solid material that is suitable for conducting analyte detection. Materials which may be used will include various plastic polymers and copolymers, such as polypropylenes, polystyrenes, polyimides, and polycarbonates. Inorganic materials such as glass and silicon are also useful. Silicon is especially advantageous in view of its high thermal conductivity, which facilitates rapid heating and cooling of the device if necessary. The substrate may be formed from a single material or from a plurality of materials.

The sample-distribution network is formed by any suitable method known in the art. For plastic materials, injection molding will generally be suitable to form detection chambers and connecting channels having a desired pattern. For silicon, standard etching techniques from the semiconductor industry may be used, as described in Sze (1988), for example.

Typically, the device substrate is prepared from two or more laminated layers, as will be discussed below with reference to Figs. 6A-6C to 8. For optical detection, the device will include one or more layers which provide an optically transparent window for each detection chamber, through which the analyte-specific signal is detected. For this purpose, silica-based glasses, quartz, polycarbonate, or an optically transparent plastic layer may be used, for example. Selection of the particular window material depends in part on the optical properties of the material. For example, in a fluorescence-based assay, the material should have low fluorescence emission at the wavelength(s) being measured. The window material should also exhibit minimal light absorption for the signal wavelengths of interest.

With chemiluminescence detection, where light of a distinctive wavelength is typically generated without illumination of the sample by an outside light source, the absorptive and reflective properties of the substrate will be less important, provided that the substrate provides at least one optically transparent window for detecting the signal.

Figs. 6A-6C illustrate a specific embodiment of a device in accordance with the invention. With reference to Figs. 6A and 6B, device 100 includes a sample inlet 102, sample-distribution network 104, and vacuum port 106 which is connected to the terminus of network 104. Network 104 includes a perpendicular array of detection chambers 108 (7 rows x 8 columns) linked to sample delivery channel 110 via dead-end fluid connections 112. The device further includes vertical panel 114 adjacent sample inlet 102, for attaching an identifying label to the device and as an attachment allowing the user to hold the device.

As can be seen from Fig. 6B, the detection chambers are packed closely together to increase the number of analytes which can be tested in the device. Fluid connections 112 are provided in an L-shaped configuration (Fig. 6C) to impede fluid flow out of the chambers after sample loading, and to help isolate the contents of the chambers from each other. Although the horizontal rows of detection chambers in Figs. 6A and 6B are shown as being separated from each other by variable vertical spacing (to enhance the clarity of the figures), it will be appreciated that the chambers can be separated by equal distances in both the vertical and horizontal directions, to facilitate analysis of the chambers.

Figs. 7 and 8 illustrate two exemplary approaches for forming a testing device in accordance with Figs. 6A-6B. Fig. 7 shows two substrate layers 140 and 142 which can be brought together to form sample-distribution network 104 (Fig. 6A). The network is defined primarily by substrate layer 140, which contains indentations defining a sample inlet 102 (not shown), a plurality of detection chambers 108, sample delivery channel 110, and dead-end fluid connections 112. Contact of substrate layer 142 with the opposing face of layer 140 completes the formation of network 104.

Fig. 8 shows substrate layers 150 and 152 for forming a network by another approach. Substrate layer 150 contains indentations defining a plurality of detection chambers 108. Substrate layer 152, on the other hand, contains indentations defining sample delivery channel 110 and dead-end fluid connections 112. Network 104 can then be formed by contacting the opposing faces of the two substrate layers as in Fig. 7.

Since the device is designed to provide a vacuum-tight environment within the sample-distribution network for sample loading, and also to provide detection chambers having carefully defined reaction volumes, it is desirable to ensure that the network and associate detection chambers

Further illustration of the invention is provided by the device shown in Fig. 9. Device 160 includes a network-defining substrate layer 161 and a flat substrate layer 180 for bonding with and sealing layer 161.

Layer 161 includes sample inlet 162 and indentations defining (i) a sample-distribution network 164 and (ii) vacuum reservoir 166 connected to the terminus of network 164. Network 164 includes a 2-dimensional perpendicular array of detection chambers 168 (7 x 7) linked to sample delivery channel 170 via dead-end fluid connections 172. The device further includes vertical panel 174 adjacent sample inlet 162, as in Figs. 6A-6B. Formation of network 164 is completed by contacting the entirety of the upper surface of device 160 with the opposing face of a layer 180, which is preferably provided in the form of a membrane or thin layer.

Device 160 in Fig. 9 is distinguished from device 100 in Fig. 6A in that device 160 includes a vacuum reservoir 166, instead of a vacuum port, at the terminus of delivery channel 170. In addition, sample inlet 162 in device 160 is conveniently adapted to operate in conjunction with inlet fitting 190, so that evacuation of the network and sample loading can be effected from a single site with respect to the network.

Sample inlet 162 includes a hollow inlet cylinder 176 having an open proximal end 177, which connects to network 172, and an open, distal end 178. Cylinder 176 further includes an opening 179 located near the terminus of the distal end.

Inlet fitting 190 includes an inlet cap structure 200 and a port structure 210 appended thereto. Cap structure 200 defines a hollow cylinder 202 having an open, proximal end 204 and a closed, distal end 206. The inner diameter of cylinder 202 is dimensioned to form a vacuum-tight seal when placed over inlet 162. Port structure 210 defines a vacuum port 212 and a sample port 214. Ports 212 and 214 communicate with cylinder 202 via openings 216 and 218, respectively, which are formed in the side of cylinder 202. Fitting 190 additionally includes guide structure 220 for receiving the adjacent edge of panel 174, to orient and guide fitting 190 when fitting 190 is fitted over and slided along inlet 162.

Exemplary dimensions of a device which has been prepared in accordance with Fig. 9 are the following: detection chambers 168, 1.2 mm x 1.2 mm x 0.75 mm; delivery channel 170, 0.25 mm x 0.25 mm (width x depth); dead-end fluid connection 172, 0.25 mm x 0.25 mm (width x depth); external dimensions: 22 cm x 15 cm x 1 mm (dimensions of network-defining portion, excluding inlet 162 and panel 174). Preferably, the detection chambers in the microdevice of the invention have volumes less than 10 μL, less than 2 μL, and most preferably less than or equal to 1 μL.

reagents suitable to detect or measure the analyte(s) of interest. It will be appreciated that more than one analyte can be tested for in a single detection chamber, if desired.

In one embodiment, the analytes are selected-sequence polynucleotides, such as DNA or RNA, and the analyte-specific reagents include sequence-selective reagents for detecting the polynucleotides. The sequence-selective reagents include at least one binding polymer which is effective to selectively bind to a target polynucleotide having a defined sequence.

The binding polymer can be a conventional polynucleotide, such as DNA or RNA, or any suitable analog thereof which has the requisite sequence selectivity. For example, binding polymers which are analogs of polynucleotides, such as deoxynucleotides with thiophosphodiester linkages, and which are capable of base-specific binding to single-stranded or double-stranded target polynucleotides may be used. Polynucleotide analogs containing uncharged, but stereoisomeric methylphosphonate linkages between the deoxyribonucleoside subunits have been reported (Miller, 1979, 1980, 1990, Murakami, Blake, 1985a, 1985b). A variety of analogous uncharged phosphoramidate-linked oligonucleotide analogs have also been reported (Froehler). Also, deoxyribonucleoside analogs having achiral and uncharged intersubunit linkages (Stirchak) and uncharged morpholino-based polymers having achiral intersubunit linkages have been reported (U.S. Patent No. 5,034,506). Binding polymers known generally as peptide nucleic acids may also be used (Buchardt, 1992). The binding polymers may be designed for sequence specific binding to a single-stranded target molecule through Watson-Crick base pairing, or sequence-specific binding to a double-stranded target polynucleotide through Hoogstein binding sites in the major groove of duplex nucleic acid (Kornberg). A variety of other suitable polynucleotide analogs are also known.

The binding polymers for detecting polynucleotides are typically 10-30 nucleotides in length, with the exact length depending on the requirements of the assay, although longer or shorter lengths are also contemplated.

In one embodiment, the analyte-specific reagents include an oligonucleotide primer pair suitable for amplifying, by polymerase chain reaction, a target polynucleotide region of the selected analyte which is flanked by 3'-sequences complementary to the primer pair. In practicing this embodiment, the primer pair is reacted with the target polynucleotide under hybridization conditions which favor annealing of the primers to complementary regions of opposite strands in the target. The reaction mixture is then thermal cycled through several, and typically about 20-40, rounds of primer extension, denaturation, and primer/target sequence annealing, according to well-known polymerase chain reaction (PCR) methods (Mullis, Saiki).

Typically, both primers for each primer pair are pre-loaded in each of the respective detection chambers, along with the standard nucleotide triphosphates, or analogs thereof, for primer extension

The fluorescer and quencher dyes are spaced close enough together to ensure adequate quenching of the fluorescer, while also being far enough apart to ensure that the polymerase is able to cleave the FQ-oligo at a site between the fluorescer and quencher. Generally, spacing of about 5 to about 30 bases is suitable, as generally described in Livak et al. (1995). Preferably, the fluorescer in the FQ-oligo is covalently linked to a nucleotide base which is 5' with respect to the quencher.

In practicing this approach, the primer pair and FQ-oligo are reacted with a target polynucleotide (double-stranded for this example) under conditions effective to allow sequence-selective hybridization to the appropriate complementary regions in the target. The primers are effective to initiate extension of the primers via DNA polymerase activity. When the polymerase encounters the FQ-probe downstream of the corresponding primer, the polymerase cleaves the FQ-probe so that the fluorescer is no longer held in proximity to the quencher. The fluorescence signal from the released fluorescer therefore increases, indicating that the target sequence is present.

One advantage of this embodiment is that only a small proportion of the FQ-probe need be cleaved in order for a measurable signal to be produced. In a further embodiment, the detection reagents may include two or more FQ-oligos having distinguishable fluorescer dyes attached, and which are complementary for different-sequence regions which may be present in the amplified region, e.g., due to heterozygosity (Lee, 1993).

In another embodiment, the detection reagents include first and second oligonucleotides effective to bind selectively to adjacent, contiguous regions of a target sequence in the selected analyte, and which may be ligated covalently by a ligase enzyme or by chemical means (Whiteley, 1989; Landegren, 1988) (oligonucleotide ligation assay, OLA). In this approach, the two oligonucleotides (oligos) are reacted with the target polynucleotide under conditions effective to ensure specific hybridization of the oligonucleotides to their target sequences. When the oligonucleotides have base-paired with their target sequences, such that confronting end subunits in the oligos are basepaired with immediately contiguous bases in the target, the two oligos can be joined by ligation, e.g., by treatment with ligase. After the ligation step, the detection wells are heated to dissociate unligated probes, and the presence of ligated, target-bound probe is detected by reaction with an intercalating dye or by other means.

The oligos for OLA may also be designed so as to bring together a fluorescer-quencher pair, as discussed above, leading to a decrease in a fluorescence signal when the analyte sequence is present.

In the above OLA ligation method, the concentration of a target region from an analyte polynucleotide can be increased, if necessary, by amplification with repeated hybridization and

In another embodiment, the analyte is an antigen, and the analyte-specific reagents in each detection chamber include an antibody specific for a selected analyte-antigen. Detection may be by fluorescence detection, agglutination, or other homogeneous assay format. As used herein, "antibody" is intended to refer to a monoclonal or polyclonal antibody, an Fc portion of an antibody, or any other kind of binding partner having an equivalent function.

For fluorescence detection, the antibody may be labeled with a fluorescer compound such that specific binding of the antibody to the analyte is effective to produce a detectable increase or decrease in the compound's fluorescence, to produce a detectable signal (non-competitive format). In an alternative embodiment (competitive format), the detection means includes (i) an unlabeled, analyte-specific antibody, and (ii) a fluorescer-labeled ligand which is effective to compete with the analyte for specifically binding to the antibody. Binding of the ligand to the antibody is effective to increase or decrease the fluorescence signal of the attached fluorescer. Accordingly, the measured signal will depend on the amount of ligand that is displaced by analyte from the sample. Exemplary fluorescence assay formats which may be adapted for the present invention can be found in Ullman (1979, 1981) and Yoshida (1980), for example.

In a related embodiment, when the analyte is an antibody, the analyte-specific detection reagents include an antigen for reacting with a selected analyte antibody which may be present in the sample. The reagents may be adapted for a competitive or non-competitive type format, analogous to the formats discussed above. Alternatively, the analyte-specific reagents include a mono- or polyvalent antigen having one or more copies of an epitope which is specifically bound by the antibody-analyte, to promote an agglutination reaction which provides the detection signal.

In yet another embodiment, the selected analytes are enzymes, and the detection reagents include enzyme-substrate molecules which are designed to react with specific analyte enzymes in the sample, based on the substrate specificities of the enzymes. Accordingly, detection chambers in the device each contain a different substrate or substrate combination, for which the analyte enzyme(s) may be specific. This embodiment is useful for detecting or measuring one or more enzymes which may be present in the sample, or for probing the substrate specificity of a selected enzyme. Particularly preferred detection reagents include chromogenic substrates such as NAD/NADH, FAD/FADH, and various other reducing dyes, for example, useful for assaying hydrogenases, oxidases, and enzymes that generate products which can be assayed by hydrogenases and oxidases. For esterase or hydrolase (e.g., glycosidase) detection, chromogenic moieties such as nitrophenol may be used, for example.

In another embodiment, the analytes are drug candidates, and the detection reagents include a suitable drug target or an equivalent thereof, to test for binding of the drug candidate to the

dichroism, optical rotation, Raman scattering, radioactivity, and light scattering. Preferably, the optical signal is based on fluorescence, chemiluminescence, or light absorbance.

In general, the optical signal to be detected will involve absorbance or emission of light having a wavelength between about 180 nm (ultraviolet) and about 50 μm (far infrared). More typically, the wavelength is between about 200 nm (ultraviolet) and about 800 nm (near infrared). A variety of detection apparatus for measuring light having such wavelengths are well known in the art, and will typically involve the use of light filters, photomultipliers, diode-based detectors, and/or charge-coupled detectors (CCD), for example.

The optical signals produced in the individual detection chambers may be measured sequentially by iteratively scanning the chambers one at a time or in small groups, or may be measured simultaneously using a detector which interrogates all of the detection chambers continuously or at short time intervals. Preferably, the signals are recorded with the aid of a computer capable of displaying instantaneously (in real-time) the signal level in each of the detection chambers, and also storing the time courses of the signals for later analysis.

The optical signal in each chamber may be based on detection of light having one or more selected wavelengths with defined band-widths (e.g., 500 nm $\pm$ 5 nm). Alternatively, the optical signal may be based on the shape or profile of emitted or absorbed light in a selected wavelength range. Preferably, the optical signal will involve measurement of light having at least two distinctive wavelengths in order to include an internal control. For example, a first wavelength is used to measure the analyte, and a second wavelength is used to verify that the chamber is not empty or to verify that a selected reagent or calibration standard is present in the detection chamber. An aberration or absence of the signal for the second wavelength is an indication that the chamber may be empty, that the sample was improperly prepared, or that the detection reagents are defective.

In studies conducted in support of the invention, a detection assembly was prepared for fluorescence detection of target polynucleotides in a sample using a device in accordance with the invention. The assembly includes a translation stage for positioning the test device. The test device includes a 7 x 7 array of addressable detection chambers containing fluorescent detection reagents. The detector in the assembly consists of a tungsten bulb (or quartz halogen bulb, 75 W) illumination source, a CCD camera, and appropriate focusing/collection optics. The illumination source is positioned so as to illuminate the device diagonally from above (e.g., at an inclination angle of 45 degrees with respect to the illuminated surface). The optics include two lenses separated by an emission filter. The first lens collimates the incoming image for the emission filter, and the second

for use in the invention operate on a variety of principles. In one, the analyte being measured is itself capable of interacting with an analyte-specific reagent to generate an electrochemical species, i.e., a species capable of function as an electron donor or acceptor when in contact with an electrode. As an example, reaction of the analyte cholesterol with the reagent cholesterol oxidase generates the electrochemical species H_2O_2 which, in contact with an electrode, produces a measurable current in a circuit containing the electrode.

The analyte-specific reagent may be localized on a film separated from the electrode surface by a permselective layer that is selectively permeable to the electrochemical species (and other small components in the sample). When sample fluid is added to the biosensor, reaction of the analyte with the corresponding reagent produces an electrochemical species whose presence and amount are quantitated by current measurement through the electrode.

Alternatively, the analyte-specific reagent may be a receptor which is specific for the analyte. Initially, the receptor sites are filled with an analyte-enzyme conjugate. In the presence of analyte, the conjugates are displaced from the receptor, and are then free to migrate to positions close to the electrode, for production of transient electrochemical species (such as H_2O_2 in the presence of catalase) in the vicinity of the electrode.

Another general type of biosensor employs a lipid bilayer membrane as a gate for electrochemical species interposed between a sample-fluid chamber and an electrode. The bilayer is provided with ion-channel proteins which function as ion gates that can be opened by analyte binding to the proteins. Thus, binding of analyte to the channel proteins (which serve as the analyte-specific reagent) leads to ion flow across the membrane and detectable signal at the electrode.

Thin-film biosensors of the type mentioned above may be formed in a microchip or small-substrate format by photolithographic methods, such as described in U.S. Patents Nos. 5,391,250, 5,212050, 5,200,051, and 4,975,175. As applied to the present invention, the chamber walls in the substrate may serve as the substrate for deposition of the required electrode and film layers. In addition to these layers, suitable conductive connectors connecting the electrodes to electrical leads are also laid down.

In a typical device, each chamber contains a biosensor for a given analyte. When sample is introduced into the device, the multiple sample analytes are then separately measured in the chambers, with the results being reported to a processing unit to which the device is electrically connected.

Patent 5,164,055, and Menchen et al., U.S. Patent 5,290,418), to segregate the chambers from each other, or with a reagent-containing solution which facilitates the assay.

In a particularly advantageous embodiment of the invention, a large-volume syringe can be used to generate a vacuum inside the sample distribution network of the device prior to loading. By "large-volume" is meant that the volume of the syringe is greater than the total internal volume of the device (i.e., of the sample distribution network). Preferably, the volume of the syringe is at least 20-fold greater than the interior volume of the device. With reference to the device in Fig. 9, the inlet-tip of the syringe is connected to vacuum port 212. When opening 216 is aligned with opening 179, the syringe is used to draw air from the interior of the device, thereby lowering the internal pressure. For example, if a syringe with a volume of 50 mL is used, and the internal volume of the device is 100 μL, the pressure in the sample distribution network can be reduced by a factor of 500 (= 0.1 mL/50 mL). Thus, an initial internal pressure of 760 torr can be reduced to less than 2 torr. With reference to the device in Fig. 6A, the syringe can be connected to fitting 106 or 102 using appropriate connections, to withdraw air from the distribution network.

Accordingly, the present invention includes a kit comprising (i) a device as described above and (ii) a syringe for drawing air from the interior of the device. The invention also includes a method of using the kit to detect one or more analytes in a sample, as described above. It will be appreciated that using a syringe greatly simplifies the step of creating a vacuum inside the device, so that the device can be used quickly or immediately without needing a mechanical vacuum pump.

V. Utility

The present invention can be used in a wide variety of applications. The invention can be used for medical or veterinary purposes, such as detecting pathogens, diagnosing or monitoring disease, genetic screening, determining antibody or antigen titers, detecting and monitoring changes in health, and monitoring drug therapy. The invention is also useful in a wide variety of forensic, environmental, and industrial applications, including screening drug candidates for activity.

More generally, the present invention provides a convenient method for simultaneous assay of multiple analytes in a sample. The invention is highly flexible in its applications, being adaptable to analysis of a wide variety of analytes and sample materials. By providing pre-dispensed, analyte-specific reagents in separate detection chambers, the invention eliminates the need for complicated and time-consuming reagent preparation.

Practice of the invention is further simplified since the detection chambers can be loaded via a single sample inlet. The use of uniformly sized detection chambers renders the device self-

10x "TAQMAN" buffer A:

500 mM KCl, 100 mM Tris-HCl, 0.1 M EDTA, 600 nM Passive Reference 1 (ROX), pH 8.3 at room temperature, autoclaved

$MgCl_2$:

20 mM $MgCl_2$ in autoclaved deionized ultrafiltered water

A description of the sequences of the forward primer, the reverse primer, and the TAMRA-labeled probe can be found in PE Applied Biosystems "TAQMAN" PCR Reagent Protocol (1996), which also describes the general steps of the "TAQMAN" assay technique. The forward and reverse primers were effective to produce a 297 basepair PCR product.

A flat substrate layer 180 and a substrate layer 161 were formed from polycarbonate by standard injection-molding methods (substrate layer 161) or from sheet stock (layer 180). The volume of each detection chamber was 1 μL.

Detection chambers were loaded with different amounts of forward primer, reverse primer, and fluorescent probe as follows. To a polypropylene tube was added 0.5 mL each of ß-actin forward primer solution, reverse primer solution, and fluorescent probe, to give a final primer/probe stock solution volume of 1.5 mL. This solution was then loaded into alternating detection chambers in substrate layer 161 using a robotically controlled microsyringe. Specifically, alternate chambers were loaded with either a 1x, 5x or 10x amount of primer/probe solution, with 1x (14 nL primer/probe stock solution) being equivalent to a final concentration in a detection chamber of 15 nM of each primer and 10 nM of fluorescent probe (after the dried chamber is subsequently filled with sample), 5x (72 nL primer/probe stock solution) being equivalent to a final concentration of 75 nM of each primer and 50 nM of fluorescent probe, and 10x (145 nL primer/probe stock solution) being equivalent to a final concentration of 150 nM of each primer and 100 nM of fluorescent probe. The amounts of primer and probe in the loaded chambers corresponded to 1/20, 1/4, and 1/2 of the concentrations used under standard reaction conditions, for the 1x, 5x, and 10x chambers, respectively. The loaded chambers produced a "checkerboard" pattern in substrate layer 161 where each loaded chamber was separated by an intervening empty chamber.

After the loaded chambers were allowed to air-dry to dryness at room temperature, the loaded substrate layer (161) was joined to a flat substrate layer 180 by ultrasonic welding. Inlet fitting 190 was then placed over sample inlet 162, such that opening 179 was aligned with vacuum port opening 216. The sample distribution network 164 and detection chambers 168 were evacuated via vacuum port 212, which was connected to a vacuum pump, to a final internal pressure of approximately 1 to 10 torr.

background for the chambers which did not contain ß-actin primer and probe, indicating that there was no cross-contamination between detection chambers after 40 heat/cool cycles.

The highest final fluorescence signals were obtained in detection chambers loaded with a 10x amount of primers and probe, with detectable signals appearing after about 23 cycles. The 5x chambers also showed detectable signals after cycle 23, but the final fluorescence signal was not as high as that for the 10x wells (due to lower probe concentration). Thus, the ß-actin gene was readily detected using primer and probe concentrations equal to 1/4 and 1/2 of those used under ordinary conditions. The results also show that the preloaded primers and probes were successfully dissolved in the sample after sample loading.

Although the invention has been described by way of illustration and example for purposes of clarity and understanding, it will be appreciated that various modifications can be made without departing from the invention. All references cited above are incorporated herein by reference.

8. The device of claim 1, wherein said detection means includes an optically transparent window associated with each detection chamber, through which such signal can be optically detected.

9. The device of claim 1, wherein the analytes are polynucleotides, and at least two of said detection chambers each contain detection reagents for detecting different polynucleotide analytes.

10. The device of claim 9, wherein the analyte-specific detection reagents include first and second oligonucleotide primers having sequences effective to hybridize to opposite end regions of complementary strands of a selected polynucleotide analyte segment, for amplifying the segment by primer-initiated polymerase chain reaction.

11. The device of claim 10, wherein said analyte-specific detection reagents further include a fluorescer-quencher oligonucleotide capable of hybridizing to the analyte segment in a region downstream of one of the primers, for producing a detectable fluorescent signal when the analyte is present in the sample.

12. The device of claim 9, wherein the analyte-specific detection reagents include first and second oligonucleotides effective to bind to adjacent, contiguous regions of a target sequence in the selected analyte, for oligonucleotide ligation assay detection of the analyte.

13. The device of claim 12, wherein the analyte-specific detection reagents include a second pair of oligonucleotides which are effective to bind to adjacent, contiguous regions complementary to the regions bound by the first pair of oligonucleotides, for amplification of the polynucleotide analyte by ligase chain reaction.

14. The device of claim 9, wherein the analyte-specific detection reagents include a binding polymer effective to hybridize to a selected sequence in the polynucleotide analyte, and the binding polymer includes a fluorescent dye moiety which produces a detectable signal upon hybridizing to the selected sequence.

15. The device of claim 9, wherein the analyte-specific reagents additionally include an intercalating compound which produces an optically detectable signal upon intercalating a double-stranded polynucleotide.

24. The method of claim 21, wherein said channel means includes an individual channel for each detection chamber, for providing a dead-end fluid connection between said inlet and each detection chamber.

25. The method of claim 21, wherein said device further includes a vacuum port connected to the channel means at a site in fluid communication with the sample inlet and detection chambers, and prior to said applying, said network is evacuated by applying a vacuum to the vacuum port.

26. The method of claim 25, wherein said vacuum port is connected to the channel means at a site that is downstream of said sample inlet and said detection chambers.

27. The method of claim 21, wherein said device further includes a non-flowthrough vacuum reservoir in fluid communication with said channel means.

28. The method of claim 21, wherein the analytes are polynucleotides, and at least two of said detection chambers each contain detection reagents for detecting different polynucleotide analytes.

29. The method of claim 28, wherein the analyte-specific reagents include an oligonucleotide primer pair suitable for amplifying, by polymerase chain reaction, an analyte segment which is flanked by sequences complementary to the primer pair.

30. The method of claim 29, wherein said analyte-specific detection reagents further include a fluorescer-quencher oligonucleotide capable of hybridizing to the analyte segment in a region downstream of one of the primers, for producing a detectable fluorescent signal when the analyte is present in the sample.

31. The method of claim 28, wherein the analyte-specific detection reagents include first and second oligonucleotides effective to bind to adjacent, contiguous regions of a target sequence in the selected analyte, for oligonucleotide ligation assay detection of the analyte.

32. The method of claim 31, wherein the analyte-specific detection reagents include a second pair of oligonucleotides which are effective to bind to adjacent, contiguous regions complementary

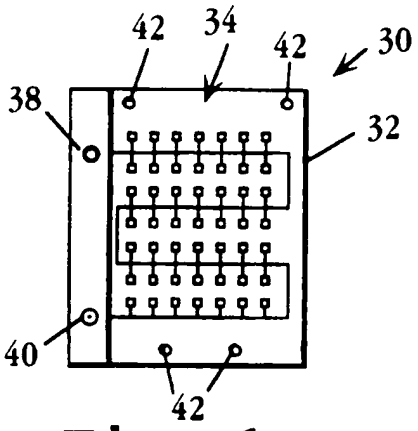


Fig. 1A

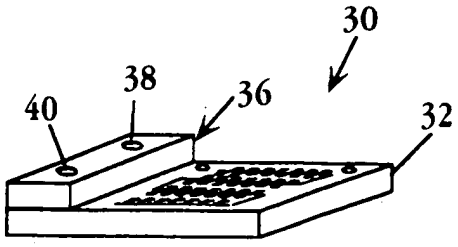


Fig. 1B

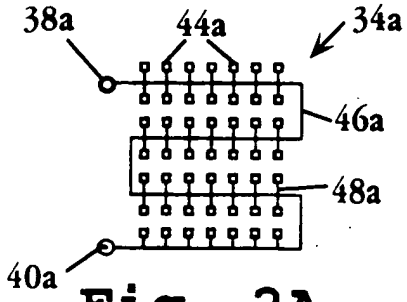


Fig. 2A

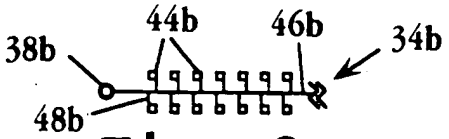


Fig. 2B

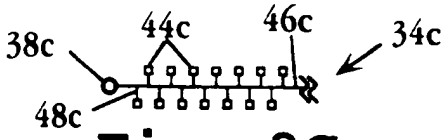


Fig. 2C

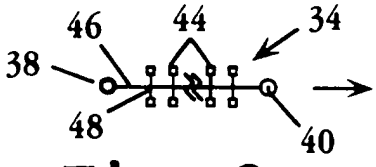


Fig. 3A

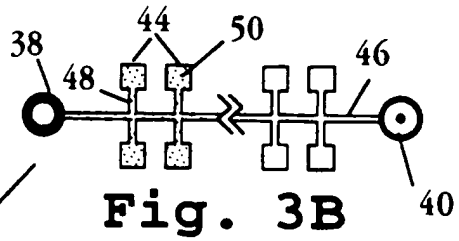


Fig. 3B

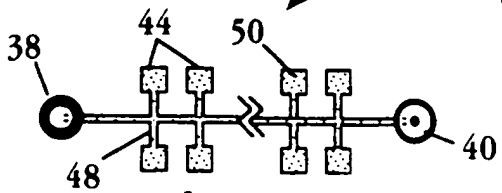


Fig. 3C

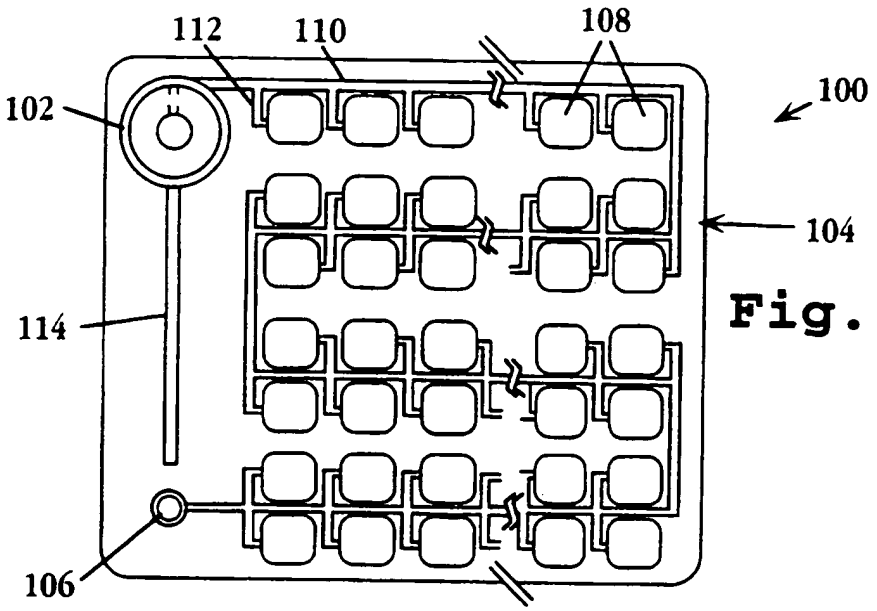


Fig. 6B

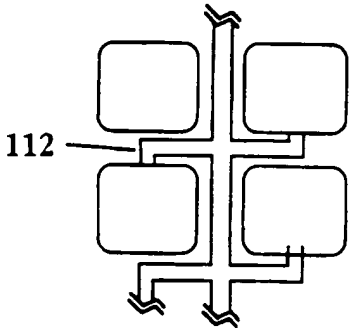


Fig. 6C

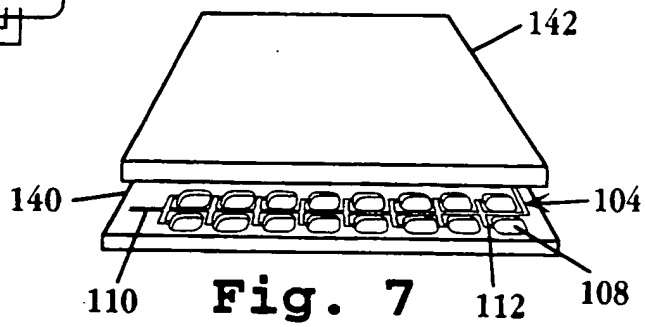


Fig. 7

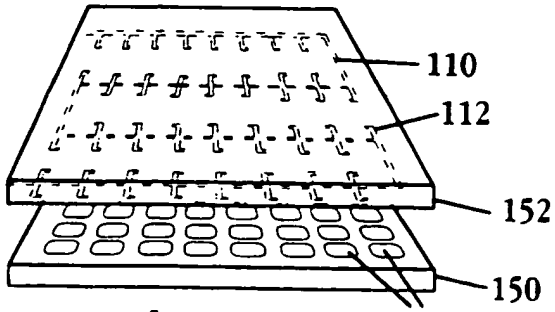


Fig. 8

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 97/05458

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 B01L3/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 B01L

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 94 11489 A (BIOLOG INC) 26 May 1994	1
A	see page 3 - page 12	2

Y	EP 0 131 934 A (PANDEX LABORATORIES INC) 23 January 1985	1
A	see abstract	5-8

A	WO 95 21382 A (R.E. FIELDS) 10 August 1995	1,4-10, 14-18, 20,21, 24-38
	see page 6 - page 18	
	see page 22 - page 23	
	see page 39 - page 43	
	see figures 1-12	

	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

'A' document defining the general state of the art which is not considered to be of particular relevance

'E' earlier document but published on or after the international filing date

'L' document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

'O' document referring to an oral disclosure, use, exhibition or other means

'P' document published prior to the international filing date but later than the priority date claimed

'T' later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

'X' document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

'Y' document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

'&' document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
18 July 1997	18.08.97

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+ 31-70) 340-3016	De Kok, A

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inte ...nal Application No
PCT/US 97/05458

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9411489 A	26-05-94	EP 0672110 A	20-09-95
		JP 8503544 T	16-04-96
		US 5589350 A	31-12-96
EP 0131934 A	23-01-85	US 4704255 A	03-11-87
		DE 3485333 A	23-01-92
		JP 1860814 C	27-07-94
		JP 60100054 A	03-06-85
WO 9521382 A	10-08-95	AU 1745495 A	21-08-95
		CA 2182513 A	10-08-95
		EP 0752105 A	08-01-97
WO 9603206 A	08-02-96	US 5595712 A	21-01-97
		EP 0772490 A	14-05-97
EP 0496200 A	29-07-92	US 5182082 A	26-01-93
		AT 137798 T	15-05-96
		AU 640838 B	02-09-93
		AU 8965791 A	30-07-92
		DE 69210424 D	13-06-96
		DE 69210424 T	05-12-96
		ES 2086556 T	01-07-96
		JP 4315946 A	06-11-92
		JP 8012135 B	07-02-96
		NZ 240604 A	27-06-94
		US 5338666 A	16-08-94